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(71) Applicant *(for all designated States except US)*: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; John B. Gorsiraweg 14, Curacao (AN).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EL TAYAR, Nabil [CH/US]; 143 Gerald Road, Milton, MA 02186 (US). BLECHNER, Steven [US/US]; 40 Harestwood Drive, West Bridgewater, MA 02379 (US). JAMESON, Brad [US/US]; 76 Robbins Street, Milton, MA 02186 (US). TEPPER, Mark [US/US]; 60 Eagle Drive, Canton, MA 02021 (US).

(74) Agent: BROWDY, Roger, L.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street N.W., Washington, DC 20004 (US).

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(54) Title: CD28/CTLA–4 INHIBITING PEPTIDOMIMETICS, PHARMACEUTICAL COMPOSITIONS THEREOF, AND METHOD OF USING SAME

(57) Abstract

The present invention relates to peptidomimetics capable of inhibiting CD28 and/or CTLA–4 interaction with CD80 (B7–1) and CD86 (B7–2) and having a core amino acid sequence, LeuMetTyrProProProTyrTyr, corresponding to residues 2 to 9 of SEQ ID NO:1. The present invention also relates to pharmaceutical compositions and a method of treating pathologies and disorders which are improved by inhibition of CD28 and/or CTLA–4 interaction with CD80 and CD86.

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CD28/CTLA-4 INHIBITING PEPTIDOMIMETICS, PHARMACEUTICAL COMPOSITIONS THEREOF, AND METHOD OF USING SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to peptidomimetics, capable of inhibiting CD28 and/or CTLA-4 interaction with CD80 (B7-1) and CD86 (B7-2), and pharmaceutical compositions thereof. It also relates to the use of the peptidomimetic for the preparation of pharmaceutical compositions active in pathologies requiring CD28 and/or CTLA-4 agonism or antagonism and method of treating such pathologies.

Description of the Background Art

The interaction of antigen presented in the context of MHC class II to the T Cell Antigen Receptor Complex (TCR) provides the primary signal to the Helper T Cell to initiate clonal proliferation. Optimal T cell activation, however, requires a co-stimulatory signal in addition to the engagement of the TCR. Although several co-stimulatory molecules have been implicated in initiating the "second signal", it has become apparent that one of the major signals is provided by the interaction of CD28 with B7 molecules (CD80 and CD86) presented on the surface of the antigen presenting cell (see Figure 1).

Cell surface CD28 is a 201 amino acid glycoprotein member of the Ig-superfamily of proteins (Aruffo and Seed, 1987). It is found naturally as a homodimer and expressed constitutively on the surface of 80% of human T cells (all $CD4^+$ cells and on about 50% of the $CD8^+$ cells) and on virtually all murine T cells (Linsley and Ledbetter, 1993). Engagement of CD28 by its natural ligand B7-1 or B7-2 (CD80, CD86) results in a second signal to the T cell and an increase of IL-2 production along with down-regulation of the CD28 with respect to mRNA levels and cell surface expression (Linsley et al., 1993). The second signal is believed to be crucial for the commitment of antigen specific T cell to proliferate. Interference with this second signal in the

presence of the first signal (TCR signal) results in antigen specific T cell anergy (unresponsiveness) (Linsley et al., 1992). During the period that CD28 is down-modulated, a closely related glycoprotein, CTLA-4, is concomitantly up-regulated (Freeman et al., 1992). It is generally thought that CD28 delivers the positive costimulatory signal for growth and differentiation, while CTLA-4 is responsible for a subsequent negative signal of the cellular activation events (for a review see Lenschow et al., 1996).

Both CD28 and CTLA-4 bind to the B7 family of proteins, most notably B7-2 and B7-1 (Azuma, et al., 1993). With regard to B7-1, it is known that CTLA-4Ig binds with a 20-100 fold higher affinity than CD28Ig (Linsley et al., 1991).

Freshly isolated human and murine B cells express low levels of B7-2 but not B7-1, however the levels of both B7's are up-regulated upon activation (Hathcock et al., 1994).

In vitro studies have demonstrated that blockade of T cell co-stimulation via the CD28 signaling pathway results in the development of antigen-specific T cell anergy (Harding et al., 1991; Boussiotis et al., 1993; Linsley et al., 1991).

CTLA-4Ig has been used in a wide variety of animal models to study the *in vivo* efficacy of blocking the CD28 signaling pathway. The first *in vivo* studies showed that CTLA-4Ig was capable of suppressing humoral responses to a T cell dependent antigen (Linsley et al, 1992).

Other studies have demonstrated that blocking the CD28 costimulatory signal is effective in preventing xenograft rejection (Lenschow et al., 1992), cardiac allograft rejection (Turka et al., 1992; Lin et al., 1993), murine systemic lupus (Finck et al, 1994; Chu et al., 1996), graft versus host disease (GVHD) (Wallace et al., 1995), and experimental allergic encephalomyelitis (EAE) (Cross et al., 1995; Perrin et al., 1995; Arima et al., 1996).

Administration of CTLA-4Ig at the time of allogeneic transplantation prolongs the graft survival but fails to prevent rejection (Turka et al., 1992). If one delays the

administration of the CTLA-4Ig until 2 days after the transplant, then long-term survival of the allograft is observed as well as tolerance toward subsequent challenge with alloantigen *in vivo* (Lin et al., 1993; Sayegh et al., 1995).

Judge et al. (1996) recently studied the *in vivo* mechanism of action of CTLA-4Ig and found that delayed administration of the protein resulted in an 80-90% reduction in Th1-type cytokines and blunted the expansion of antigen specific T cells by 50%. Thus, CTLA-4Ig may be able to regulate the balance between Th1- and Th2-type responses.

In conclusion, there is ample evidence that blockade of the CD28 costimulatory pathway may be a useful therapeutic target for immune modulation. CTLA-4Ig is currently in Phase II clinical trials in psoriasis patients. However its practical use for chronic immunotherapy is limited by it being only parenterally administrable and requiring mg/kg doses.

A small molecule mimetic of CTLA-4/CD28 would have great clinical and commercial advantages and represents a long felt need.

Site-directed mutagenesis studies with both CTLA-4 and CD28 have implicated a hexapeptide stretch including several key sites in the CDR3 region of the protein, MetTyrProProProTyr (SEQ ID NO:31), as a critical contact site in the interaction with B7 (Peach et al., 1994).

European patent application EP 682,039 discloses that CTLA-4Ig fusion proteins block the interaction with B7 antigen. It also discloses CTLA-4 mutants, in which any of the amino acids, including the sequence MetTyrProProProTyr (SEQ ID NO:31), has been replaced by Ala.

International patent application WO 95/33770 is generally directed to ligands for T cell surface molecules, especially CTLA-4, which induces antigen specific apoptosis of activated T cell. Isolated peptides containing CTLA-4 fragments, constituting the epitope for such binding, are also disclosed and claimed. Such epitopes include the amino sequence ProProTyrTyrLeu (SEQ ID NO:32) (partially

overlapping with the above reported hexapeptide MetTyrProProProTyr (SEQ ID NO:31).

Scientists at Glaxo have recently attempted to use both linear as well as conformationally restrained peptides to mimic this region (Ellis et al., 1996). The Glaxo study, however, failed to yield any productive leads.

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SUMMARY OF THE INVENTION

It is an object of the invention to overcome the deficiencies of the related art, such as noted above, by providing biologically active peptidomimetics of CD28 or CTLA-4.

Accordingly, the present invention provides for peptidomimetics of CD28 or CTLA-4 which are capable of inhibiting CD28 and/or CTLA-4 interaction with CD80 (B7-1) and CD86 (B7-2). The peptidomimetics of the present invention contain a core sequence corresponding to amino acid residues 2 to 9 of SEQ ID NO:1 and may be cyclized and may include additional amino acid residues N-terminal and/or C-terminal to this core sequence.

The present invention also provides for a pharmaceutical composition which includes the peptidomimetics according to the present invention, and pharmaceutically acceptable excipients.

Further provided in the present invention is a method of treating pathologies and disorders which are improved by inhibition of CD28 and/or CTLA-4 interaction with CD80 and CD86.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates the involvement of CD28 in Helper T cell Activation.

Figure 2 shows the amino acid sequence alignment of CD28 (SEQ ID NO:33), CTLA-4 (SEQ ID NO:34), and REI (SEQ ID NO:35) used for the molecular modeling of CD28/CTLA-4.

Figure 3 shows a molecular model in ribbon diagram form of the CTLA-4 homodimer based on the REI Template.

Figure 4 shows the amino acid sequence alignment of the B7-1 protein (SEQ ID NO:36) with an IgG heavy chain of the MCO antibody (SEQ ID NO:37).

Figure 5 shows a ribbon diagram of a CTLA-4 Monomer/B7-1 protein complex.

Figure 6 shows a bar graph of an evaluation of AT 199, 200 and 201 peptides in a Human MLR(A vs B).

Figure 7 shows a bar graph of an evaluation of AT 199, 200 and 201 peptides in a Human MLR (B vs A).

Figure 8 shows a bar graph of an evaluation of AT 199 cyclized and linear peptides in a Human MLR (A vs B).

Figure 9 shows a bar graph of an evaluation of AT 199 and 201 peptides in a murine MLR.

Figure 10 shows a bar graph of a toxicity test of AT 199 and 200 peptides in THP-1, Jurkat and PBLs cells.

Figure 11 shows a bar graph of the effect of AT 199 and 200 peptides on PHA-mediated stimulation of PBL.

DETAILED DESCRIPTION OF THE INVENTION

The biologically active peptidomimetics of CD28 or CTLA-4 according to the present invention have been designed using a molecular modeling strategy, based on a "skin and bones" type design, as discussed below.

Both CD28 and CTLA-4 proteins are homodimeric Ig superfamily members consisting of a single Ig variable domain. A homology search of the Brookhaven database of known structures was conducted and a crystallized example of an Ig homodimer (code - REI) was found. The sequence alignment used in the modeling studies is shown in Figure 2.

The models of both CTLA-4 and CD28 were constructed using side chain replacements of the REI template. Because

the length of the CDR3 loop in REI is two amino acids shorter than the corresponding region on either the CD28 or CTLA-4, a CDR3 loop of appropriate length (obtained from the Brookhaven database, PDB1JHL.ENT) was grafted on to the template molecule. The newly constructed model was minimized to convergence using a conjugate-gradient function. Five cycles of annealed dynamics (iterations of an energy-dependent simulation of molecular motion followed by an energetic minimization) were used to optimize the structure prior to a final minimization step. A ribbon diagram of the CTLA-4 homodimer is shown in Figure 3.

The molecular model of the B7-1 molecule was constructed in an analogous fashion to the strategy used for CD28/CTLA-4. The best alignment found from a search of the Brookhaven database was the Ig heavy chain variable and constant domain of the MCO antibody. The sequence alignment used in building the B7-1 model is shown in Figure 4.

After the model of B7-1 was constructed, a tentative model of the CTLA-4 (CD28)/B7-1 complex was built. Several groups have published extensive site-directed mutagenesis studies that were aimed at defining the CTLA-4/B7 surfaces involved in complex formation (Peach et al., 1994, 1995; Guo et al., 1995). These mutations were aligned on the modeled proteins and used as a guide in forming the homodimeric binding complex. For simplicity, Figure 5 shows only the monomeric CTLA-4 molecule interacting with B7-1.

In the engineering of the peptidomimetics of CD28 or CTLA-4 as inhibitors of the CD28 costimulatory pathway, the molecular models described above were used as design templates. The peptidomimetics according to the present invention, which are capable of inhibiting CD28 and/or CTLA-4 interaction with CD80 (B7-1) and CD86 (B7-2) molecules have a core amino acid sequence, LeuMetTyrProProProTyrTyr, corresponding to amino acid residues 2 to 9 of SEQ ID NO:1. In addition to this core amino acid sequence, the peptidomimetic may have additional amino acid residues N-terminal to and/or C-terminal to amino acid residues 2 to 9 of SEQ ID NO:1.

Preferably, there is a Cys residue both immediately N-terminal and immediately C-terminal to the core amino acid sequence, as shown by SEQ ID NO:1. It is also preferrable that the petpidomimetic of the present invention be cyclized. When the cyclized peptidomimetics of the present invention includes the sequence of SEQ ID NO:1, the cyclization of the peptide preferably occurs via a Cys-Cys disulfide bridge between residue 1 (Cys) and 10 (Cys) of SEQ ID NO:1. Otherwise, the peptidomimetic can be cyclized with a linker, such as a synthetic chemical linker, that bridges residue 2 (Leu) and residue 9 (Tyr) of the core amino acid sequence which corresponds to residues 2 to 9 of SEQ ID NO:1. Thus, cyclization preferably occurs within one amino acid of residue 2 and residue 9 of SEQ ID NO:1. Other examples of synthetic chemical linkers include, but are not limited to, a Lys-Asp salt bridge, lanthanide cyclization, N-terminal to C-terminal cyclization, those chemical linkers disclosed in Olson et al. (1993), etc.

It is also preferred in the peptidomimetics of the present invention that up to eleven additional amino acid residues are present N-terminal to, and/or up to eleven additional amino acids are present C terminal to the core sequence corresponding to residues 2 to 9 of SEQ ID NO:1. More preferably, there are two additional amino acid residues both N-terminal and C-terminal to the sequence of SEQ ID NO:1, wherein these additional amino acid residues are positively charged amino acid residues. The amino acid sequence of SEQ ID NO:2 is an example of a preferred embodiment of a cyclized peptidomimetic of the present invention where cyclization occurs via a Cys-Cys disulfide bridge between residues 3 and 12 of SEQ ID NO:2.

The peptidomimetics according to the present invention may further include one or more of the amino acid sequences selected from SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14; SEQ ID NO:19, and SEQ ID NO:20, which can be linked to each other and/or to the sequence of SEQ ID NO:1 or the sequence corresponding to residues 2 to 9 of SEQ ID NO:1 either directly or through a suitable synthetic chemical

linker. The peptidomimetics of the present invention is also intended to encompass those peptidomimetics in which one or more bioisoteric fragments, such as are commonly used in drug design, are present in combination with or in place of the additional amino acids N-terminal and/or C-terminal to the core amino acid sequence.

It is noteworthy that the peptidomimetics of the present invention are not fragments of CTLA-4/CD28 molecules, but have been specifically designed and subsequently selected from among a number of possibilities based on the results of the biological tests reported in the Examples section.

The strategy employed here was to identify and exploit the potential contact surfaces of the CD28/CTLA-4 proteins with B7 as well as to select a region of the CD28/CTLA-4 molecule responsible for mediating homodimer formation as a means of potentially disrupting the appropriate presentation of the homodimers.

Six regions of the modeled CD28/CTLA-4 molecules were identified as potential targets (see Table 1) for peptide design.

TABLE 1: Engineered Peptides studied

Peptide	Description	Sequence	SEQ ID NO:
AT 119.1	CD28 N-terminal region	KILVKQS	3
AT 120.1	CD28 N-terminal region	KYLVKQS	4
AT 121.1	AT119.1 with C-term. acid	KILVKQS-OH	3
AT 122.1	AT120.1 with C-term. acid	KYLVKQS-OH	4
AT 125.1	CTLA-4 N-terminal region	HVEQPA-OH	5*
AT 136.1	CTLA-4 N-terminal region	HVAQPA	5
AT 137.1	CTLA-4 N-terminal region	HVEQPA	6
	CDR1 Loop		
AT 106.1	CD28 CDR-1 region	KCSYNLFSREFC	7*
AT 107.1	CD28 CDR-1 region	CKYSYNLFSREFC	8
AT 118.1	CTLA-4 CDR-1 region	ECASPGKATEVC	9
AT 123.1	CTLA-4 CDR-1 region	CEYASPGKATEVC	10
	40-45 Loop		
AT 108.1	CD28 40-45 loop	CKGLDSAVEC	11
AT 116.1	CD28 40-45 loop	LDSAVEV	12
AT 117.1	AT116.1 with C-term. acid	LDSAVEV-OH	12
AT 124.1	CTLA-4 40-45 loop	ADSQVTEV-OH	13
AT 127.1	CTLA-4 40-45 loop	ADSQVTEV	13
AT 128.1	CTLA-4 40-45 loop	CRQADSQVTEC	14*
	60-65 Loop		
AT 115.1	CD28 60-65 loop	CSKTGFNC	15
AT 197.1	CTLA-4 60-65 loop	NECTFCDD	16
AT 131.1	CTLA-4 60-65 loop	CDDSIC	17
	70-75 Loop		
AT 132.1	CTLA-4 70-75 loop	CSSGNQVC	18
AT 133.1	CTLA-4 70-75 loop	CSSPNQVC	19*
AT 135.1	CTLA-4 70-75 loop	CSPNQC	20
	CDR3 MYPPPY		
AT 109.1	CD28 CDR-3 region	CMYPPYLRGGKC	21
AT 110.1	CD28 CDR-3 region	CMYPPPYGKC	22
AT 111.1	CD28 CDR-3 region	CMYPPQYGKC	23
AT 112.1	CD28 CDR-3 region	CMYPPPYKAKC	24
AT 113.1	CD28 CDR-3 region	CKIEVMYPPPYC	25
AT 114.1	CD28 CDR-3 region	CKIEVMYPPPYLC	26
AT 129.1	CTLA-4 CDR-3 region	CMYPPPYYRGGKC	27
AT 130.1	CTLA-4 CDR-3 region	CMYPPPYYKAKC	28
AT 199.1	CTLA-4 CDR-3 region hybrid	RKCLMYPPPYYCHH	2*
AT 200.1	CTLA-4 CDR-3 region hybrid	RKCLAYPPPYYCHH	29
AT 201.1	CTLA-4 CDR-3 region hybrid	RKCLGYPPPYYCHH	30

*Note: Peptides that have shown, at least, some biological activity in the MLR assay.

The first region corresponds to the amino terminus of the protein. This is an elongated (β-strand) stretch of amino acids and is not amenable to the introduction of conformational restraints. The amino terminus is predicted to lie directly below the CDR3 region and to form part of the B7 contact surface.

The second region corresponds to the CDR1 analogous portion of the protein. The CDR1 domain has been shown via site-directed mutagenesis studies to be involved in the binding to B7 (Peach et al., 1994). Because the CDR1 region does not form a tight β-turn, but rather forms a loosely formed loop, it is difficult to design conformationally restrained peptides that closely resemble this region. The strategy employed here was to give maximal flexibility to the restrained peptides designed from this region.

The third set of peptides was made to the loop region between residues 40-45 which represents one of the major contact sites predicted to hold together the homodimer. Because the functional CD28/CTLA-4 expressed on the cell surface is predominately in the form of a homodimer (some evidence exists that there may be some involvement of monomeric presentation to B7), the objective with these analogs was to disrupt the homodimer formation.

The fourth set of peptides was derived from the 60-65 loop (SEQ ID NOs:15, 16 and 17) which is not predicted to be a part of the B7 contact surface, but constitutes a major surface exposed loop. According to the present model of interaction, peptides designed from this region should not possess any biologic activity. If any of these analogs were to display inhibitory activity, then it is evidence that the present model interaction was incorrect.

The loop formed by residues 70-75 is predicted to be directly involved in the contact with B7. Three analogs were synthesized from this region (see Table 1, SEQ ID NO: 18, 19 and 20). AT 132 represents the restrained native sequence. Modeling of this analog suggested that the intended loop was not stably formed. AT 133 was designed to correct this instability by introducing a proline (a relatively rigid,

turn promoting residue) to replace the highly flexible glycyl residue. AT 135 is a shorter analog of AT 133, which is intended to probe the contribution of residues that flank the central portion of the 70-75 loop.

Finally, the CDR3 analogous region of CTLA-4/CD28 was exploited (SEQ ID NOs: 21-30). Single site-directed mutations in this region of CTLA-4Ig completely abrogate the binding to B7 (Peach et al., 1994). Analog design from this region, however, presented some formidable engineering problems. The central hexapeptide sequence from this region is MetTyrProProProTyr (SEQ ID NO:31). These are relatively hydrophobic residues and the triple proline stretch is conformationally rigid. In terms of the molecular model of the CTLA-4/B7 complex, this region is predicted to be part of a deep contact. Experience has indicated that the most effective inhibitors of protein-protein interactions tend to require an electrostatically active "guide" sequence which mimics part of the initial "handshake" of the binding event. Consequently, a variety of different approaches were used in the engineering of the CDR3 panel of analogs. Analysis of the surface area around the CDR3 region of the protein indicated that it is surrounded by a positively charged potential. Therefore, lysine, arginine and histidine were incorporated toward the ends of the analogs to mimic this positive potential and to aid in the solubility properties of the synthesized peptide.

In the specific instance of AT 199, a hybrid analog was designed. Several different strategies were incorporated in to the design of this analog. First, four highly charged (positive) residues were incorporated adjacent to the cysteines flanking both the amino and carboxy termini of the analog. Since hydrophobic residues tend to move away from hydrophilic residues, this design was also intended to partition the MetTyrProProProTyr (SEQ ID NO:31) loop away from the positively charged residues and help force the correct formation of the CDR3 turn. The flanking residues were selected to mimic residues that are spatially juxtaposed to the CDR3 region in the native protein and are

involved in the binding to B7. They also represent important sites of electrostatic contact. The arginine at the amino terminus of the analog is a mimic of Arg33 in the native protein. The histidine residue at the carboxy terminus of the peptide is intended to mimic His2 at the amino terminus of the protein which lies directly below the CDR3 region.

Peptides AT 200 and AT 201 were designed as controls for AT 199. Previously, Peach at el. (1994) have shown that a single substitution of the methionine residue to an alanine residue in the CDR3 region results in complete abrogation of the binding of CTLA-4Ig to B7. Therefore, AT 200 is identical in sequence to AT 199, with the exception that Met is replaced by an Ala residue. AT 201 is also identical in sequence to AT 199, with the exception that Met is replaced by a flexible Gly residue.

The peptidomimetics of the present invention may be prepared by any well known procedure in the art, in particular, by the well established chemical synthesis procedures utilizing automated solid-phase peptide synthesizers followed by chromatographic purification. More particularly, the procedures disclosed in the Examples section may be followed for the preparation and preferably the cyclization of such peptides.

The pharmaceutical composition for treating pathologies and disorders which are improved by inhibition of CD28 and/or CTLA-4 interaction with CD80 and CD86 according to the present invention contains a substantially purified peptidomimetic as an active ingredient. Depending on whether the peptidomimetic includes one or more of the amino acid sequences of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, and SEQ ID NO:20, the pharmaceutical composition according to the present invention may further include one or more separate peptides having an amino acid sequence selected from SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, and SEQ ID NO:20 which are not already present in the peptidomimetic.

Examples of pathologies and disorders in which the peptidomimetics according to the present invention can be

advantageously used as a prophylactic, a therapeutic or a diagnostic are immune system diseases and cancer. Specific non-limiting examples include autoimmune diseases, such as psoriasis, multiple sclerosis, lupus erythematosus, diabetes, rheumatoid arthritis, and therapy for transplant rejection including solid organ and cellular transplants.

Further objects and advantages of the invention will be evident in the following description.

An embodiment of the invention is the administration of a pharmacologically active amount of the peptide of the invention to subjects at risk of developing pathologies and disorders which are improved by inhibition of CD28 and/or CTLA-4 interaction with CD80 and CD86 or to subjects already showing such pathologies and disorders.

Any route of administration compatible with the active principle can be used, but particularly preferred is the parenteral administration because systemic effects can be achieved in a short period of time. Parenteral administration may be by a number of different routes including, but not limited to, subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intracerebral, intranasal, oral, transdermal, or buccal routes.

It is understood that the dosage of peptide to be administered will be dependent upon the age, sex, health, weight, kind of concurrent treatment, and frequency of treatment. The dosage will be tailored to an individual patient as is understood and determinable by one of skill in the art. The dosage can be between 0.1 and 20 mg/Kg body weight, and preferably between 0.1 and 1 mg/Kg body weight.

The pharmaceutical composition for parenteral use including the active principle and a suitable vehicle can be prepared in injectable form. Vehicles for the parenteral administration are well known in the art and include, for example, water, saline solution and physiologic buffers. The vehicle can contain smaller amounts of excipients in order to maintain the solution stability and isotonicity.

The preparation of the pharmaceutical compositions can be carried out according to the ordinary modalities, and

preferably, the peptide content will be in the range between 10 mg/ml and 1,000 mg/ml.

The invention will now be described by means of the following Examples and accompanying figures, which should not be construed as in any way limiting the present invention.

Example 1: Peptide Synthesis

34 peptides were synthesized using standard Fmoc procedures as described below, where the abbreviations are as follows:

Acetonitrile (ACN), Benzyl (BZL), tert-Butyloxy-carbonyl (BOC), Dichloromethane (DCM), Diisopropylethylamine (DIEA), Dimethyl Formamide(DMF), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), 9-Fluorenylmethyloxycarbonyl (FMOC), 2-[1H-Benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU), 1-hydroxybenzotriazole (HOBt,), N-methyl morpholine (NMM), N-methyl pyrrolidone (NMP), 2,2,5,7,8-Pentamethyl-chroman-6-sulfonyl (PMC), tert-Butyl (tBu), Triphenylmethyl (TRT), Trifluoroacetic acid (TFA), Hepta-fluorobutaric acid (HFBA).

Resins

The primary resin used is a Rink Amide Methylbenzyl-hydrilamine resin, which is a standard support for synthesizing peptides with a C-terminal amide. For peptides needing a C-terminal free carboxylic acid ending, Wang resins with the first Fmoc amino acid attached are used. The Wang resins, which contain a p-benzyloxybenzyl handle, are the standard supports for the preparation of peptide acids by the Fmoc solid phase batch synthesis strategy. Both types of resins were purchased from NovaBiochem.

Amino Acids used in Synthesis

Fmoc Amino Acids	Protecting Group
Ser, Thr, Asp, Glu	tBu
Cys, Asn, Gln, His	Trt
Arg	PMC
Lys	BOC
Trp	BOC
Ala, Gly, Ile, Met, Leu, Pro, Phe, Val	None

Chain Assembly

Protected peptide chains are initially assembled using FMOC strategy on an Applied Biosystem, Inc. Model 431A Peptide Synthesizer or a Rainin Symphony Multiple Peptide Synthesizer. Both synthesizers utilize base-labile FMOC N-terminal protected amino acids with appropriate side chain protection groups, 20% piperidine for N-terminal deprotection and HBTU for amino acid activation and coupling.

Cleavage/Extraction

The standard cleavage cocktails used for removing side chain protecting groups and releasing the peptide from the resin: Mixture A: 95% TFA, 5% Deionized Water.

For peptides without arginine, methionine, tryptophan or amino acids with the trityl protecting group (cysteine, histidine, asparagine, glutamine): Mixture B: 82.5% TFA, 5% phenol, 5% D.I. water, 5% thioanisole, 2.5% ethanedithiol.

For peptides containing arginine or methionine: Mixture B': 87% TFA, 4.3% D.I. water, 4.3% thioanisole, 4.3% ethanedithiol.

An alternative cocktail for peptides with arginine or methionine: Mixture C: 95% TFA, 2.5% D.I. water, 2.5% ethanedithiol.

For peptides without arginine or methionine and containing tryptophan or the trityl protecting group: the cleavage reaction is performed by using 100 mg - 1 g of

peptide-resin placed into a 20 ml glass vessel and cooled in an ice bath. The cleavage cocktail is prepared and also cooled in an ice bath, then added to the peptide-resin for a final volume of approx. 10 ml. The vessel is removed from the ice bath and allowed to warm to room temperature. The vessel is capped and the reaction mixture stirred at room temperature for 1.5 hours. After 1.5 hours, the solution is vacuum filtered through a medium-to-coarse porosity filter into approx. 30 ml of cold MTBE (methyl-t-butyl ether). The reaction vessel is washed with 1 ml TFA and filtered through the same filter funnel into the cold MTBE. The entire suspension is then transferred to a 50 ml centrifuge tube and centrifuged for approx. 10 minutes at 2000 rpm at room temperature. The supernatant is aspirated, the precipitate resuspended in 40 ml cold MTBE and centrifuged again. This step is repeated once more. The final supernatant is aspirated and the precipitate is treated with nitrogen to evaporate most of the remaining ether. The peptide is then dissolved in 20-30 ml of aqueous 1% -10% Acetic Acid, diluted to approx. 100-150 ml with deionized water, shell frozen, and lyophilized.

Example 2: Cyclization

Peptides designed with two cysteines for disulfide bond cyclization are processed in one of these two ways. If the crude peptide is shown by analytical HPLC to be at least 65% pure without any significant secondary peaks (>20% of main product), the peptide is first cyclized, then purified. This comprises about 90% of the peptides produced at the facility. If the crude peptide has significant secondary deletion products, it is initially purified, then cyclized, and then re-purified.

The method of cyclization is disulfide bond formation by air oxidation. 25 mg-100 mg of crude peptide is first dissolved in deionized water at a ratio of 6-10 ml/mg peptide. While stirring, the pH of the solution is raised to approx. 8.3 with 1.0 M NH_4HCO_3 (pH 8.5). The solution is stirred overnight at room temperature, with sufficient

stirring to create a vortex that reaches near the bottom of the vessel. The next day (approx. 18-24 hrs.), peptide cyclization is checked by analytical reverse-phase HPLC for characteristic changes in retention time and a 280 nm absorbance due to the disulfide bond. The solution is then lyophilized and stored or purified by direct loading onto a preparative reverse-phase HPLC column.

Purification

1. Reverse Phase Preparative HPLC

Note: peptides that tend to be more hydrophobic in nature are purified using HFBA instead of TFA to improve the chromatographic resolution of the final product.

Conditions: System - Waters Delta Prep 4000

Column - Vydac reverse-phase C18, 10 μm, 2.2 x 25 cm (Cat No. 218TP1022)

Buffers - A: Water/ 0.1%TFA B: Acetonitrile/ 0.1%TFA

Flow Rate - 15 ml/minute

Detection - Waters 484 UV detector, 220 nm

Gradient - Variable, usually 0.33% B/min up to 1.0% B/min

Lyophilized crude peptides are prepared by dissolving 50-100 mg of peptide in 200 ml of aqueous 0.1% TFA. Cyclized peptides already in solution at pH 8-8.5 are first quenched with neat TFA to lower the pH to the 2-3 range. The peptide solution is then loaded directly onto the preparative column through the "A" buffer reservoir line and the gradient program started. Collected fractions are run overnight on an autosampler analytical HPLC system. Overlapping fractions judged to be >95% pure by peak integration are pooled and lyophilized.

Sep-Pak purification

Conditions: Equipment - Baker Solid Phase Extraction

12 port Manifold

Columns - Waters Vac 12 cc 2 gram Sep-Pak columns

Buffers - H_2O/0.1% TFA

20%, 30%, 50%, 99.9% Acetonitrile/0.1% TFA solutions

Crude lyophilized peptides are prepared by dissolving 15-25 mg peptide in 8 ml of aqueous 0.1% TFA. Sep-Pak columns are first conditioned with 30 ml 99.9% Acn/0.1% TFA, followed by 30 ml of H_2O/0.1%TFA. Peptide solutions are loaded, followed by another wash of H_2O/0.1%TFA, then eluted with either 20% or 30% Acn/0.1%TFA buffers. A final wash with 50% Acn/0.1%TFA is performed to ensure complete elution and for comparison. The load volume, H_2O wash, 20%-30% Acn and 50% Acn volumes are collected separately and checked on analytical HPLC. Eluted peptide solutions are then diluted 3:1 with deionized H_2O and lyophilized.

Example 3: <u>Characterization</u>

1. Analytical Reverse-phase HPLC (For check of homogeneity of final product)

Conditions: System - Waters 500 pumps, 717 Autosampler, 490

Multiwavelength UV Detector

Column - Vydac C 18,5 μm, 0.46 x 25 cm (Cat. No. 218TP54)

Buffers - A: H_2O/ 0.1% TFA B: ACN/ 0.1% TFA

Flow Rate - 1 ml/minute

Detection - 214 nm, 280 nm

Gradient - 2 % B/minute

Purified lyophilized peptide samples are prepared by dissolving 0.2 - 1.0 mg of peptide in aqueous 0.1% TFA to a concentration of 0.5 - 1.0 mg/ml. 15 - 18 μl are injected onto the column and eluted with a gradient program of 0 - 50% ACN in 25 minutes. Chromatogram data is collected and stored with the Waters Expert-ease software system.

2. Mass Spectrometry (for checking homogeneity and covalent structure)

System: Perseptive Biosystems Voyager Elite

Type: MALDI-TOF (Matrix assisted laser desorption/ionization Time-of-flight)

Matrix: alpha-Cyano 4-hydroxy cinnamic acid (Sigma, C-2020), 10 mg/ml in 67% ACN/0.1% TFA

Peptide samples are prepared at 1 - 10 μmol conc. in 50% ACN/0.1% TFA. 0.5 μl of peptide sample, followed by 0.5 μl of matrix solution, is applied to analysis plate wells and allowed to dry. The analysis plate is loaded into the machine and the samples scanned and analyzed using a reflectron delayed-extraction method. For each sample, a cumulative data signal from 32 - 128 laser shots is collected and analyzed. Each run includes a sample well with a standard peptide for calibration.

3. Ellman's Reagent Test (To check disulfide bond cyclization)

Disulfide bond cyclization of peptides containing Tryptophan or Tyrosine cannot be checked by HPLC UV detection at 280 nm due to the high absorbance at that wavelength. The Ellman's reagent test for the presence of free sulphydryl groups of the Cysteine sidechain is an alternative indicator of disulfide bond formation.

Peptides are prepared at 0.5 mmol concentrations in reaction buffer (0.1 M sodium phosphate, pH 8). Ellman's reagent, DTNB, is prepared at a 4 mg/ml conc. and a standard of Cysteine hydrochloride monohydrate at a 0.5 mmol concentration in the same reaction buffer. 250 μl of sample, 50 μl of Ellman's reagent and 2.5 ml of reaction buffer are mixed and incubated at room temperature for 15 minutes. A blank sample of reaction buffer and a standard sample of cysteine is also tested. A yellow color indicates the presence of free sulphydryl groups.

Example 4: Human Mixed Lymphocyte Response (MLR)

Isolation of PBLS: Whole blood from donors was obtained from Interstate Blood Bank, Memphis, TN. Blood specimens were handled in a biosafety level 2 containment facility as recommended for potentially infectious blood specimens in the CDC/NIH manual Biosafety in Microbiological and Biomedical Laboratories; 3rd edition, 1993, page 10. Peripheral blood

mononuclear cells (PBMC) were separated from red blood cells and granulocytes by ficoll-hypaque purification. Whole peripheral blood was diluted 1:2 in PBS and 30 ml was overlayed onto 15 ml of ficoll-hypaque in 50 ml polypropylene tubes. The tubes were spun at 400 x g for 30 min at 25°C. Following centrifugation, the interface between the upper plasma layer and the lower ficoll layer was collected, cells washed in RPMI-1640 2x, and viable counts determined using trypan blue. Cells to be used as responders in the assay were stored on ice until mitomycin C treatment of stimulator cells was completed.

Mitomycin C treatment of stimulators

PBMC, isolated as described above, to be used as stimulators in the assay were adjusted to 2-4 x 10^6 cells/ml in complete medium (RPMI 1640 containing 10% heat-inactivated human AB serum, 2 mM glutamine, 50 μM 2-mercaptoethanol and 100 U/ml penicillin-100 μg/ml streptomycin), and treated with mitomycin C (25 μg/ml) for 30 min in a 37°C water bath. Following treatment, cells were washed with 5 volumes of complete medium 3x, and viable counts determined using trypan blue. To set up autologous stimulation controls some responder cells were also treated with mitomycin C as mentioned above.

CTLA-4Ig and peptides

Purified CTLA-4Ig fusion protein was produced, as described in Steurer et al. 1995, from a NS-1 cell line kindly donated by Dr. T. Strom (Beth Israel Hospital, Boston, MA) as a 1 mg/ml solution in sterile PBS. The protein was stored frozen at -80°C. Upon thawing, the aliquot was stored at 4°C. Purified peptides were lyophilized according to known procedures. Peptides were reconstituted in sterile PBS, pH 7.4 at 2 mM concentration, aliquoted in microfuge tubes and stored frozen at -20°C. For the assay, an aliquot of the peptide was thawed and diluted to 200 μM in complete medium.

Mixed Lymphocyte Response Assay (Human)

For the one-way allogeneic mixed lymphocyte response (MLR) assay, responder cells were plated at 10^5 cells/well, and stimulator cells at 5 x 10^4 cells/well in 96-well round bottom plates. Cells were incubated with serial dilutions of the anti-CD4 Ab Leu 3A (1 μg/ml - 0.06 μg/ml) in triplicate, or an isotype-matched control Ab. Cyclosporin A at 1 μg/ml was used as an additional control.

CTLA-4 Ig was serially diluted and tested from 10 μg/ml to 0.15 μg/ml. Peptides were tested either directly or in the presence of a constant spiked-in dose of CTLA-4 Ig at 0.5 μg/ml. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 7 days.

Proliferation of the cells was determined by pulsing the wells with ^{3}H-Tdr (1 μCi/well) for the last 18 h of the assay. Plates were harvested using a Tomtec plate harvester and the counts incorporated determined using a Wallac microbetaplate plus reader.

Mitogen Stimulation Assays

For these assays, ficoll-hypaque purified PBMC (10^5 cells/well) were incubated in flat-bottom 96-well tissue culture plates with indicated concentrations of phytohemagglutinin (PHA; 5, 2.5, 1.25, 0.5 μg/ml) for 3 days at 37°C in a humidified 5% CO_2/air incubator. Cells were incubated in the presence or absence of various concentrations of CTLA-4 Ig, purified anti-CD80 mAb (1 μg/ml), anti-CD86 mAb (1 μg/ml), and peptides 199 and 201 serially diluted from 100 μM to 12.5 μM for the period of the assay.

Proliferation of the cells was determined by pulsing the wells with ^{3}H-Tdr (1 μCi/well) for the last 6 h of the assay. Plates were harvested using a Tomtec plate harvester and the counts incorporated determined using a Wallac microbetaplate plus reader.

Example 5: Murine MLR

One-way murine MLRs were set up using the C57Bl/6 mice splenocytes as stimulators and BalB/c splenocytes as responders.

Isolation of mouse splenocytes

Spleens were excised from mice (Jackson Laboratories, Bar Harbor, ME) 6-8 weeks of age. Cells were separated from the capsule using sterile frosted glass slides and washed in cold RPMI 1640 1x. Red blood cells were lysed by treating the spleen cell suspension with cold Tris ammonium chloride buffer (2 ml/spleen) for 3 min on ice. After lysis, the cells were washed 2x in 5 volumes of complete medium (RPMI 1640 containing 10% heat-inactivated FBS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin-100 μg/ml streptomycin, 1 mM sodium pyruvate and 1 mM non-essential amino acids).

Plastic-adherent cells were removed from splenocytes of BalB/c mice by adjusting the cell concentration to 3 x 10^6 cells/ml in complete medium and incubating the cells for 1.5-2 h at 37°C in T-75 flasks. Following incubation, non-adherent cells were collected by gently washing the flasks and the percent recovery determined (60-70%). Bal b/C responder cells were stored on ice until mitomycin C treatment of stimulator cells was completed.

Mitomycin C treatment of stimulators

Following RBC lysis splenocytes of C57Bl/6 mice were adjusted to 2-4 x 10^6 cells/ml in complete medium and treated with mitomycin C (50 μg/ml) for 30 min at 37°C in a humidified 5% CO_2 in air atmosphere. Following treatment, cells were washed with 5 volumes of complete medium 3x, and viable counts determined using trypan blue.

To set up autologous stimulation controls some responder cells were also treated with mitomycin C as mentioned above.

Mixed Lymphocyte Response Assay (Murine)

For the assay, responder and stimulator cells were plated at 105 cells/well in 96-well round bottom plates. Cells were incubated with serial dilutions of an anti-CD4 Ab (50 ng/ml - 0.05 ng/ml) in triplicate, or an isotype-matched control Ab. Cyclosporine A at 1 μg/ml was used as an additional control. CD28/CTLA-4 peptides 199 and 201 were serially diluted and tested at a final concentration of 100 μM to 1.56 μM. CTLA-4 Ig serially diluted from 10 μg/ml was used as a positive control for inhibiting the MLR.

Appropriate scrambled peptide controls and matrix controls were included in every assay. The plates were incubated at 37°C in a humidified 5% CO2 in air atmosphere for 4 days. Proliferation of the cells was determined by pulsing the wells with 3H-Tdr (1 μCi/well) for the last 6 h of the assay. Plates were harvested and the counts incorporated determined as described above.

Example 6: Biological Screening of the Peptides in the Human MLR AND CTLA-4Ig Binding Assay

A total of 34 peptides were tested for inhibition of lymphocyte proliferation in the human MLR. Each peptide was tested in a MLR at doses ranging from 12.5 to 100 μM. The activity of each peptide was identified by assigning a + and - to the peptide depending on its ability to inhibit lymphocyte proliferation in the MLR (cpms).

A - was assigned if all concentrations of peptide tested gave <15% change in cpm.

A +, ++, or +++ was assigned depending on the degree of inhibition caused by the peptide, where:

+ was assigned when the concentration of peptide tested gave >20% change in cpm;

++ was assigned if the peptide tested gave >25% change in cpm in a dose dependant fashion; and

+++ was assigned if the peptide tested gave >50% change in cpm in a dose dependent fashion. In addition to the MLR, these 34 peptides were also evaluated in a CTLA-4Ig/B7 binding assay in which the ability of these peptides

to affect the binding of CTLA-4Ig to B7 on Cess B cells was examined by FACS analysis.

As with the MLR, a + or - was assigned to each peptide depending on its ability to effect CTLA-4Ig binding to Cess B cells; where - represents no change in binding, + represents a minor change of <10% in binding, and a ++ represents a change of > 20% in binding. The results of the MLR and B7 binding analysis are shown in Table 3.

Table 3: Overview of MLR/Binding Data with the Peptide Panel

Peptide Number	Human MLR 1/24, 1/31, 3/6, 4/17, 5/2	Binding Assay 3/24, 3/25, 4/5, 4/25
AT 106.1	++, +, +, +/-	++, ++
AT 107.1	-, +, +, -	+/-
AT 108.1	-, -, -	-
AT 109.1	+/-	-, +
AT 110.1	-	-, -
AT 111.1	-, +/-	-
AT 112.1	-	-, -
AT 113.1	-	-, -
AT 114.1	Toxic	Toxic
AT 115.1	-	-
AT 116.1	-	-
AT 117.1	-	+
AT 118.1	-	Not tested
AT 119.1	-, +/-	-
AT 120.1	-, +/-	-
AT 121.1	+/-, -	+/-
AT 122.1	-, -	+, -
AT 123.1	-, -	+/-, -
AT 124.1	-, -	+, -
AT 125.1	-, -	-, +

AT 127.1	-, -	+/-, +/-
AT 128.1	++, -	+
AT 129.1	-, -	++
AT 130.1	-, -	-, -
AT 131.1	+, -, -	+/-
AT 132.1	+, -, -	+, -
AT 133.1	++, +	++, -
AT 134.1		
AT 135.1	+/-, -	++
AT 136.1	++, +, -	+++, +
AT 137.1	-, +	+/-, -
AT 197.1	-, -, -	-, -, -
AT 199.1	+, +, +	+, +, +
AT 200.1	-, -, -	-, -, -
AT 201.1	-, -, -	-, -, -

The data from each peptide was analyzed without bias, such that the results from prior assays were not considered in determining whether a peptide was assigned a + or a -.

As can be seen from the table above, only a small number of peptides exhibited activity in either of these assays. Those peptides that exhibited activity in either the MLR or the binding assay (AT#s:106, 107, 128, 131, 132, 133, 135, 136, 199) were retested several times to determine if the initial observation was reproducible.

Based on the cumulative results of this analysis, peptides which exhibited semi-reproducible inhibition in either the MLR or the binding assay, or both, were selected for further analysis. The selected peptides were AT 106, 128, 133, 135, and 136, and later, peptide AT 199.

Analysis of Positive Peptides from Primary Screening (AT 106, 128, 133, 135, and 136)

Since peptides AT 106, 128, 133, 135, and 136 showed some activity in the MLR and/or binding assays during primary screening, these peptides were selected for further analysis. These peptides were retested in the MLR and binding assay either alone or in combination. In two MLRs, none of these peptide by themselves had any consistent effect on the MLR at concentrations up to 200 μM. Analysis of these same peptides in the Cess B binding assay showed no effect on CTLA-4Ig binding.

Identification and Evaluation of AT 199

During the course of our screening effort, the last 3 peptides tested were peptides 199, 200, and 201. The results of two human MLRs comparing the activity of AT 199, 200, and 201 in the dose range of up to 100 μM are shown in Figure 6 and 7.

As can be seen in one MLR AT 199 inhibited lymphocyte proliferation by ~70% whereas in the other MLR it inhibited ~30%. The degree of lymphocyte proliferation (30,000 cpm) was the same in both assays. Though we have no explanation for the lower inhibition of AT 199 in this MLR, similar trends have been observed for CTLA-4Ig and may be a function of the state of the cells used to generate the MLR. That AT 199 did not inhibit both MLRs as effectively is suggestive of this peptide not being cytotoxic and that the activity of AT 199 is biologically significant. AT 199 was tested in a total of 6 human MLR assay and was active in 5 out of 6.

The mass spectrometer analyses of each of these analogs showed that the expected mass was obtained, however, AT 199 contained a significant portion of linear product (32%).

In order to show that the observed activity of AT 199 was not due to an artifact of synthesis and to distinguish whether the refolded analog or the free linear form of the analog is responsible for the inhibitory behavior seen in the MLR, three new batches of AT 199 were produced.

AT 199.2A was a resynthesis of AT 199.1, where the refolding conditions were prolonged to 5 days and the pH of the refolding buffer raised from pH 8.5 to pH 10.0 to aid in the efficiency of the procedure.

AT 199.2B was treated with iodoacetamide in order to purify a purely linear analog. This procedure modifies the free sulphydryls through a simple alkylation of the sulfurs such that the cysteines are not sterically hindered but are also neither reactive nor available for forming a disulfide bridge.

AT 199.3 was a resynthesis of AT 199.1 using the original protocols without change. Table 4 shows a summary of the AT 199 analogs synthesized and the results of a quantitative Ellman's reaction to ascertain the balance between covalently cyclized and free linear populations.

Table 4: Results of the free sulphydryl content of the AT 199 Analogs

Peptide	% Cyclized vs. Linear
AT 199.1	68% cycl.: 32% lin.
AT 199.2A	94.1% cycl.: 5.9% lin.
AT 199.2B	0% cycl.: 100% lin.
AT 199.3	98.4% cycl.: 1.6% lin.

The newly synthesized batches of AT 199 were used to more specifically address whether linear or cyclized populations of AT 199 are responsible for the activity seen with this analog (note - it is unclear why the AT 199.3 cyclized more efficiently than AT 199.1, since identical procedures were used). The population of free sulphydryl-containing linear peptide was reduced by more than an order of magnitude in the AT 199.3 analog relative to the original batch. If the activity observed in the previous MLR's were due to free linear analogs, then one would expect to see a dramatic reduction in activity in the AT 199.3. The inhibitory profile associated with the alkylated AT 199.2B

should be able to tell us whether or not the observed activity is related to conformational specificity.

Evaluation of AT 199.3, 199.2A and 199.2B in the Human MLR

As described above, another synthesis of AT 199 was conducted (199.3) along with the generation of a linear (199.2B) and a fully cyclized (199.2A) version of AT 199. These peptides were evaluated in different human MLRs in comparison to peptide AT 201 and in the presence and absence of CTLA-4Ig (0.5 μg/ml).

AT 199.3 and 199.2A inhibited the MLR 30% and 50 %, respectively, at 100 μM in one MLR, and by 70% and 25%, respectively, in another MLR. AT 199.2B, on the other hand, inhibited < 10% in the MLR's at the highest concentration (100 μM) tested. From this result we conclude that AT 199 requires a cyclized conformation to be active.

These peptides were also tested for activity in other 2 MLRs in which CTLA-4Ig was spiked in at 0.5 μg/ml. The results of one of these studies are shown in Figure 8. AT 199.3, when added with 0.5 μg/ml of CTLA-4Ig, inhibited the MLR over and above CTLA-4Ig by itself. AT 199.2A also was additive when added in combination with CTLA-4Ig, but AT 199.2B and peptide 201 had no effect when added in combination with CTLA-4Ig. These results suggest that the cyclized AT 199 has activity at inhibiting the human MLR and can enhance the effect of CTLA-4Ig.

Evaluation of AT 199 in a Murine MLR

The primary sequence of CTLA-4 is very similar between human and mouse. A murine CTLA-4Ig was used throughout these studies to inhibit a human MLR. Therefore, activity of AT 199 was tested on a murine MLR. The results of this study are shown in Figure 9.

AT 199.3 inhibited the murine MLR ~85% which was similar to the inhibition by 10 μg/ml of CTLA-4Ig itself. Peptide 201 in this same assay inhibited only ~25%. Thus, it appears that like CTLA-4Ig , AT 199 is active in both the human and mouse system.

Toxicity Evaluation of AT 199 and 201 Peptides

To determine if the activity of AT 199 was due to a specific effect on lymphocyte proliferation in the MLR or due to a non-specific inhibition of lymphocyte proliferation in general (i.e., toxicity), the toxicity of AT 199 was tested against THP-1, Jurkat, and resting and activated PBLs. The results of these experiments are shown in Figure 10.

As seen in Figure 10, no inhibition of THP-1, Jurkat or resting primary PBLs was observed in the presence of 100 μM AT 199.

AT 199 was tested for inhibition of PHA activated PBLs. PHA activation, while not specifically targeted at the CD28 pathway, has some dependence on this pathway depending on the concentration of PHA used to activate lymphocytes. To determine the AT 199 of phytohemagglutinin (PHA) activation on CD28 signaling, CTLA-4Ig was included in each assay.

The effect of AT 199 on a representative PHA activation is shown in Figure 11. As can be seen in Figure 11, 10 μg/ml CTLA-4Ig inhibited PHA activation by ~40-50% at both concentrations of PHA (2.5 and 5 μg/ml). AT 199 on the other hand had only a minor effect on inhibiting PHA activation in this experiment suggesting that PHA activation of lymphocytes from this particular donor was only partially working through the CD28 pathway. Thus, AT 199 is not toxic to lymphocytes, but appears to have lower potency than CTLA-4Ig to act on the B7/B28 pathway.

AT 199, derived from the CDR3 domain, was the only peptide of the 34 analogs assayed that exhibited significant, reproducible inhibition in the MLR by itself. The inhibition was dose dependent and exhibited an apparent IC_{50} between 50-100 μM. Peach et al. (1994) have shown that a single Met to Ala substitution in the CDR3 region of human CTLA-4 abolishes the ability of the protein to bind to B7. Based on this observation, two control peptides of AT 199 were synthesized in which the Met was changed to either Ala (AT 200) or Gly (AT 201). Other than these single amino acid changes, the control peptides were identical to AT 199. These control peptides did not inhibit any of the MLRs as much as AT 199 in

which they were assayed. Thus, AT 199 appears to exhibit sequence specificity in its ability to inhibit the MLR.

AT 199 was treated with iodoacetamide in order to alkylate the sulfurs on the peptide such that a disulfide bridge could not form. This linear peptide, AT 199.2B, had no effect on the proliferation of the MLR. Thus, AT 199 appears to possess conformational specificity.

AT 199 and its control analogs were assayed for overt toxicity and non-specific inhibition. The peptides were added to growing cultures of THP-1 cells, Jurkat cells and primary peripheral blood lymphocytes (PBLs). No effect was observed on the growth of any of these cell cultures indicating that the inhibition observed in the MLR was not due to toxicity.

In order to test for an unanticipated mechanism of inhibition, AT 199 was assayed using PHA-stimulation of PBLs. Phytohemagglutinin (PHA) is a lectin extracted from the red kidney bean and is a mixture of five tetrameric glycoproteins. PHA stimulates T cells in the presence of accessory cells (but not B cells) by cross-linking a variety of critical T cell surface molecules, including CD3, CD2, CD4, CD8 and LFA-1 (Geppert, 1992). The involvement of CD28 in this stimulation is dependent on the degree of crosslinking by PHA. At the appropriate concentration of PHA, the simultaneous engagement of cell surface receptors results in a proliferation response that can bypass the need for specific costimulatory signals such as CD28. This can be monitored by examining CTLA-4Ig inhibition of PHA mitogenesis. AT 199 did not inhibit the PHA stimulated T cells under the same conditions in which the MLR was dramatically inhibited. CTLA-4Ig inhibited PHA activation slightly. Thus, this data is consistent with a biologic effect directed at a specific aspect of T cell activation.

From the data reported here using 34 peptides, it can be concluded that at least 1 peptide (AT 199) which alone inhibits a CD28 dependent human immune response has been isolated.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range

of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
(B) STREET: 14, John B. Gorsiraweg
(C) CITY: CURACAO
(E) COUNTRY: NETHERLANDS ANTILLES

(A) NAME: EL TAYAR, Nabil
(B) STREET: 143 Gerald Road
(C) CITY: Milton
(D) STATE: Massachussetts
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 02186

(A) NAME: BLECHNER, Steven
(B) STREET: 40 Harestwood Drive
(C) CITY: West Bridgewater
(D) STATE: Massachussetts
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 02379

(A) NAME: JAMESON, Brad
(B) STREET: 76 Robbins Street
(C) CITY: Milton
(D) STATE: Massachussetts
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 02186

(A) NAME: TEPPER, Mark
(B) STREET: 60 Eagle Drive
(C) CITY: Canton
(D) STATE: Massachussetts
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 02021

(ii) TITLE OF INVENTION: CD28/CTLA-4 INHIBITING PEPTIDES, PHARMACEUTICAL COMPOSITIONS THEREOF, AND METHOD OF USING SAME

(iii) NUMBER OF SEQUENCES: 37

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: BROWDY AND NEIMARK, P.L.L.C.
(B) STREET: 419 Seventh Street N.W., Ste. 300
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20004

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: YUN, Allen C
(B) REGISTRATION NUMBER: 37,971
(C) REFERENCE/DOCKET NUMBER: EL TAYAR=1 PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 628-5197
(B) TELEFAX: (202) 737-3528

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Leu Met Tyr Pro Pro Pro Tyr Tyr Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg Lys Cys Leu Met Tyr Pro Pro Pro Tyr Tyr Cys His His
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Ile Leu Val Lys Gln Ser
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Tyr Leu Val Lys Gln Ser
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

His Val Ala Gln Pro Ala
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His Val Glu Gln Pro Ala
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Cys Ser Tyr Asn Leu Phe Ser Arg Glu Phe Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys Lys Tyr Ser Tyr Asn Leu Phe Ser Ala Arg Glu Phe Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Cys Ala Ser Pro Gly Cys Ala Thr Glu Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Cys Glu Tyr Ala Ser Pro Gly Lys Ala Thr Glu Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Cys Lys Gly Leu Asp Ser Ala Val Glu Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Asp Ser Ala Val Glu Val
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala Asp Ser Gln Val Thr Glu Val
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Cys Arg Gln Ala Asp Ser Gln Val Thr Glu Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Cys Ser Lys Thr Gly Phe Asn Cys
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asn Glu Cys Thr Phe Cys Asp Asp
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Cys Asp Asp Ser Ile Cys
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Cys Ser Ser Gly Asn Gln Val Cys
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Cys Ser Ser Pro Asn Gln Val Cys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Cys Ser Pro Asn Gln Cys
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Cys Met Tyr Pro Pro Tyr Leu Arg Gly Gly Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Cys Met Tyr Pro Pro Pro Tyr Gly Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Cys Met Tyr Pro Pro Gln Tyr Gly Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Cys Met Tyr Pro Pro Pro Tyr Lys Ala Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Cys Lys Ile Glu Val Met Tyr Pro Pro Pro Tyr Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Cys Lys Ile Glu Val Met Tyr Pro Pro Pro Tyr Leu Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Cys Met Tyr Pro Pro Pro Tyr Tyr Arg Gly Gly Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Cys Met Tyr Pro Pro Pro Tyr Tyr Lys Ala Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Arg Lys Cys Leu Ala Tyr Pro Pro Pro Tyr Tyr Cys His His
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Arg Lys Cys Leu Gly Tyr Pro Pro Pro Tyr Tyr Cys His His
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Met Tyr Pro Pro Pro Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Pro Pro Tyr Tyr Leu
1 5

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ile Leu Val Lys Gln Ser Pro Met Leu Val Ala Tyr Asp Asn Ala Val
1 5 10 15

Asn Leu Ser Cys Lys Tyr Ser Tyr Asn Leu Phe Ser Arg Glu Phe Arg
20 25 30

Ala Ser Leu His Lys Gly Leu Asp Ser Ala Val Glu Val Cys Val Val
35 40 45

Tyr Gly Asn Tyr Ser Gln Gln Leu Gln Val Tyr Ser Lys Thr Gly Phe
50 55 60

Asn Cys Asp Gly Lys Leu Gly Asn Glu Ser Val Thr Phe Tyr Leu Gln
65 70 75 80

Asn Leu Tyr Val Asn Gln Thr Asp Ile Tyr Phe Cys Lys Ile Glu Val
85 90 95

Met Tyr Pro Pro Pro Tyr Leu Asp Asn Glu Lys Ser Asn Gly
100 105 110

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser Ser Arg Gly Ile
1               5                  10                  15

Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys Ala Thr Glu Val
            20                  25                  30

Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val Thr Glu Val Cys
        35                  40                  45

Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser
    50                  55                  60

Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn Leu Thr Ile Gln
65                  70                  75                  80

Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu
                85                  90                  95

Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn Gly
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp
1               5                  10                  15

Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr Leu
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
        35                  40                  45

Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Pro Tyr Thr Phe Gly
                85                  90                  95

Gln Gly Thr Lys Leu Gln Ile
            100
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Val Ile His Val Thr Lys Glu Val Lys Glu Val Ala Thr Leu Ser Cys
1               5                  10                  15

Gly His Asn Val Ser Val Glu Glu Leu Ala Gln Thr Arg Ile Tyr Trp
            20                  25                  30

Gln Lys Glu
        35
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Ser Gly Pro Gly Leu Val Lys Pro Ser Glu Ala Leu Ser Leu Thr Cys
1               5                  10                  15

Thr Val Ser Gly Asp Ser Ile Asn Thr Ile Leu Tyr Tyr Trp Ser Trp
            20                  25                  30

Ile Arg Gln Pro Pro
        35
```

WHAT IS CLAIMED IS:

1. A peptidomimetic compound, comprising amino acid residues 2 to 9 of SEQ ID NO:1.

2. The peptidomimetic compound according to claim 1, wherein the peptidomimetic compound is cyclized.

3. The peptidomimetic compound according to claim 1, wherein the peptidomimetic compound is cyclized via a linker between amino acid residues 2 and 9 of SEQ ID NO:1.

4. The peptidomimetic compound according to claim 1, further comprising 1 to 11 amino acids N-terminal to amino acid residue 2 to 9 of SEQ ID NO:1.

5. The peptidomimetic compound according to claim 4, wherein the 1 to 11 amino acids N-terminal to amino acid residues 2 to 9 of SEQ ID NO:1 are positively charged amino acids.

6. The peptidomimetic compound according to claim 1, further comprising 1 to 11 amino acids C-terminal to amino acid residues 2 to 9 of SEQ ID NO:1.

7. The peptidomimetic compound according to claim 6, wherein the 1 to 11 amino acids C-terminal to amino acid residues 2 to 9 of SEQ ID NO:1 are positively charged amino acids.

8. The peptidomimetic compound according to claim 1, further comprising one or more amino acid sequences selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, and SEQ ID NO:20.

9. The peptidomimetic compound according to claim 8, wherein the one or more amino acid sequences selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, and SEQ ID NO:20 are linked to each other and/or to amino acid residues 2 to 9 of SEQ ID NO:1 either directly or via a linker.

10. The peptidomimetic compound according to claim 1, wherein the peptidomimetic compound comprises the amino acid sequence of SEQ ID NO:1.

11. The peptidomimetic compound according to claim 10, wherein the peptidomimetic compound is cyclized via a

Cys-Cys disulfide bridge between amino acid residues 1 and 10 of SEQ ID NO:1.

12. The peptidomimetic compound according to claim 10, further comprising one or more amino acid sequences selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, and SEQ ID NO:20.

13. The peptidomimetic compound according to claim 12, wherein the one or more amino acid sequences selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14; SEQ ID NO:19, and SEQ ID NO:20 are linked to each other and/or to amino acids of SEQ ID NO:1 either directly or via a linker.

14. The peptidomimetic compound according to claim 10, further comprising 1 to 10 amino acids N-terminal to amino acid residues 1 to 10 of SEQ ID NO:1.

15. The peptidomimetic compound according to claim 14, wherein the 1 to 10 amino acids N-terminal to amino acid residues 1 to 10 of SEQ ID NO:1 are positively charged amino acids.

16. The peptidomimetic compound according to claim 10, further comprising 1 to 10 amino acids C-terminal to amino acid residues 1 to 10 of SEQ ID NO:1.

17. The peptidomimetic compound according to claim 16, wherein the 1 to 10 amino acids C-terminal to amino acid residues 1 to 10 of SEQ ID NO:1 are positively charged amino acids.

18. The peptidomimetic compound according to claim 10, further comprising two positively charged amino acids both N-terminal to and C-terminal to amino acid residues 1 to 10 of SEQ ID NO:1.

19. The peptidomimetic compound according to claim 18, wherein the peptidomimetic compound has the amino acid sequence of SEQ ID NO:2 and is cyclized via a Cys-Cys disulfide bridge between amino acid residues 3 and 12 of SEQ ID NO:2.

20. A pharmaceutical composition for treating pathologies and disorders which are improved by inhibition of CD28 and/or CTLA-4 interaction with CD80 and CD86, comprising

the cyclized peptide according to claim 1, and a pharmaceutically acceptable excipient.

21. The pharmaceutical composition according to claim 20, further comprising a peptide having the amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:14, SEQ ID NO:19, SEQ ID NO:20, and a combination thereof.

22. A method of treating pathologies and disorders which are improved by inhibition of CD28 and/or CTLA-4 interaction with CD80 and CD86, comprising administering the pharmaceutical composition according to claim 19 to a patient in need thereof.

Co-Stimulatory Signal

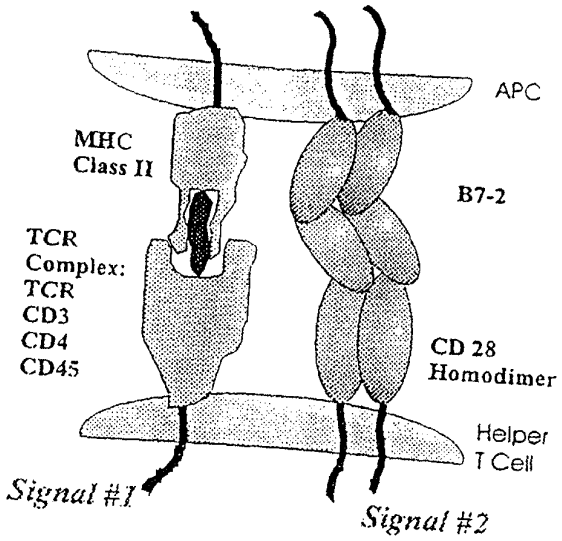


Figure 1

Alignment of the REI Ig Homodimer with CD28/CTLA-4

```
 21 ILVKQSPMLVAYDNAV..NLSCKYSYNLFSREFRASLHKGLDSAV.EVCVV CD28
  1 MHVAQPAVVLASSRGI.ASFVCEYASPGKATEVRVTVLRQADSQVTEVCAA CTLA
  2 IQMTQSPSSLSASVGDRVTITCQASQD.IIKYLNWYQQTPGKAPKLLIYEA REI

 69 YGNYSQQLQVYSKTGFNCDGKLGNESVTFYLQNLYVNQTDIYFCKIEVMY CD28
 51 TYMMGNELTFLDDS..ICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMY CTLA
 52 SNLQAG.....VPS..RFSGSGSGTDYTFTISSLQPEDIATYYC..QQYQ REI

119 PPPYLDNEKSNG CD28
 99 PPPYY.LGIGNG CTLA
 95 PYTFGQGTKLQI REI
```

Figure 2

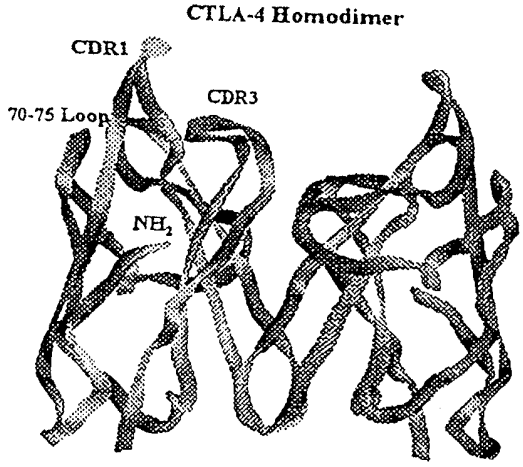


Figure 3

Sequence Alignment of B7-1 with a Heavy Chain Ig (MCO)

```
B7-1   VIHVTKEVKEVATLSCGHNVSVEELAQTRIYWQ--KE--
                 |: :|:|  :|| :::: :  ||   ::
MCO    SGPGLVKPSEALSLTC--TVSGDSINTILYYWSWIRQPP

-KKMV-LTMM--SGDMNIWPEYKNR-TI-FDITNNLSIVIL-ALRPSDEGTYECVVLKYE
 | :  :: :  ||:    |: |:| ||  ::::|     | :: ::|:::| |: :
GKGLEWIGYIYYSGSTYGNPSLKSRVTISVNTSKNQFYSKLSSVTAADTAVYYCARVPLV

KDAFKREHLAEV-TLSVKAD--FPTPSISDFEIPTSNIRRIICSTSGGFPEP-HLSWLEN
 ::: :: |::| : |:|:   || :: |  : :::::  : | ::: ||:|  :|| ::
VNPWGQGTLVTVSSASTKGPSVFPLAPSS--KSTSGGTAALGCLVKDYFPQPVTVSW-NS

GEELNAINTTVSQDPETELYAVSSKLDFNMTT--NHSFMCLIKYGHLRVNQTFNWNTTKQ
|:  ::::|  :  ::::||::|| ::   ::  :::::| ::  | : | : : : : :
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN--HKPSNTKVD-KRVAP

EHF--PDN
| :  |:
ELLGGPSV
```

Figure 4

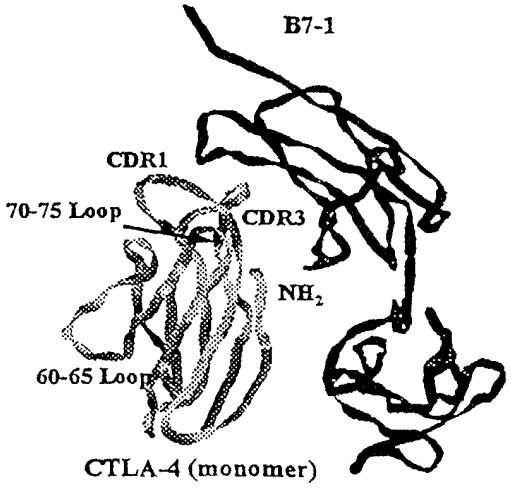


Figure 5

FIG. 6

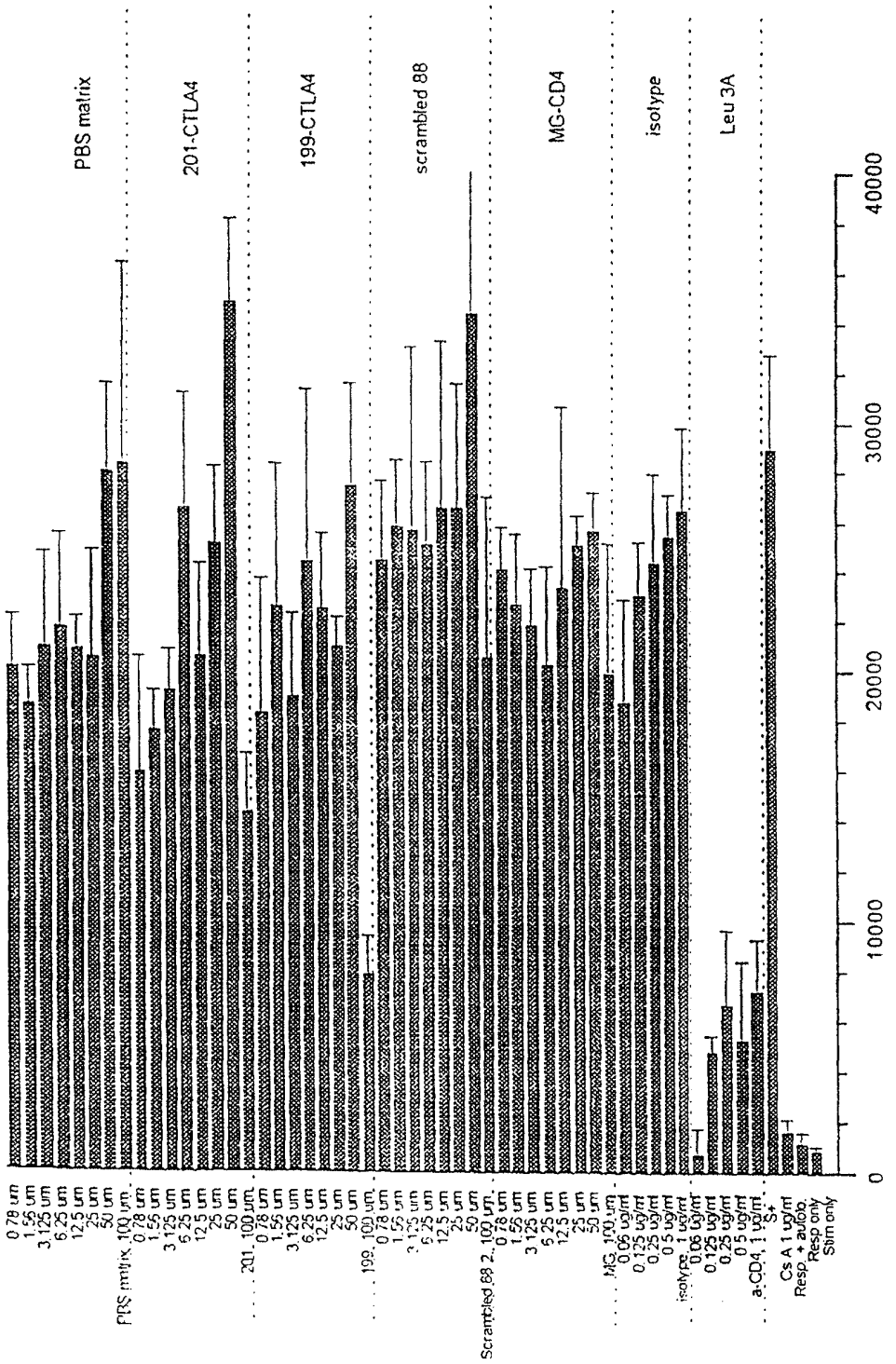

PBS matrix
201-CTLA4
199-CTLA4
scrambled 88
MG-CD4
isotype
Leu 3A
0 78 um
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
PBS matrix 100 um
201 100 um
199 100 um
Scrambled 88 2 100 um
MG 100 um
0.06 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
isotype 1 ug/ml
0.06 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
a-CD4 1 ug/ml
S+
Cs A 1 ug/ml
Resp + autol
Resp only
Stim only
0
10000
20000
30000
40000
3H-Tdr incorporation (cpm)
mean of triplicate points +/- SD

FIG. 7

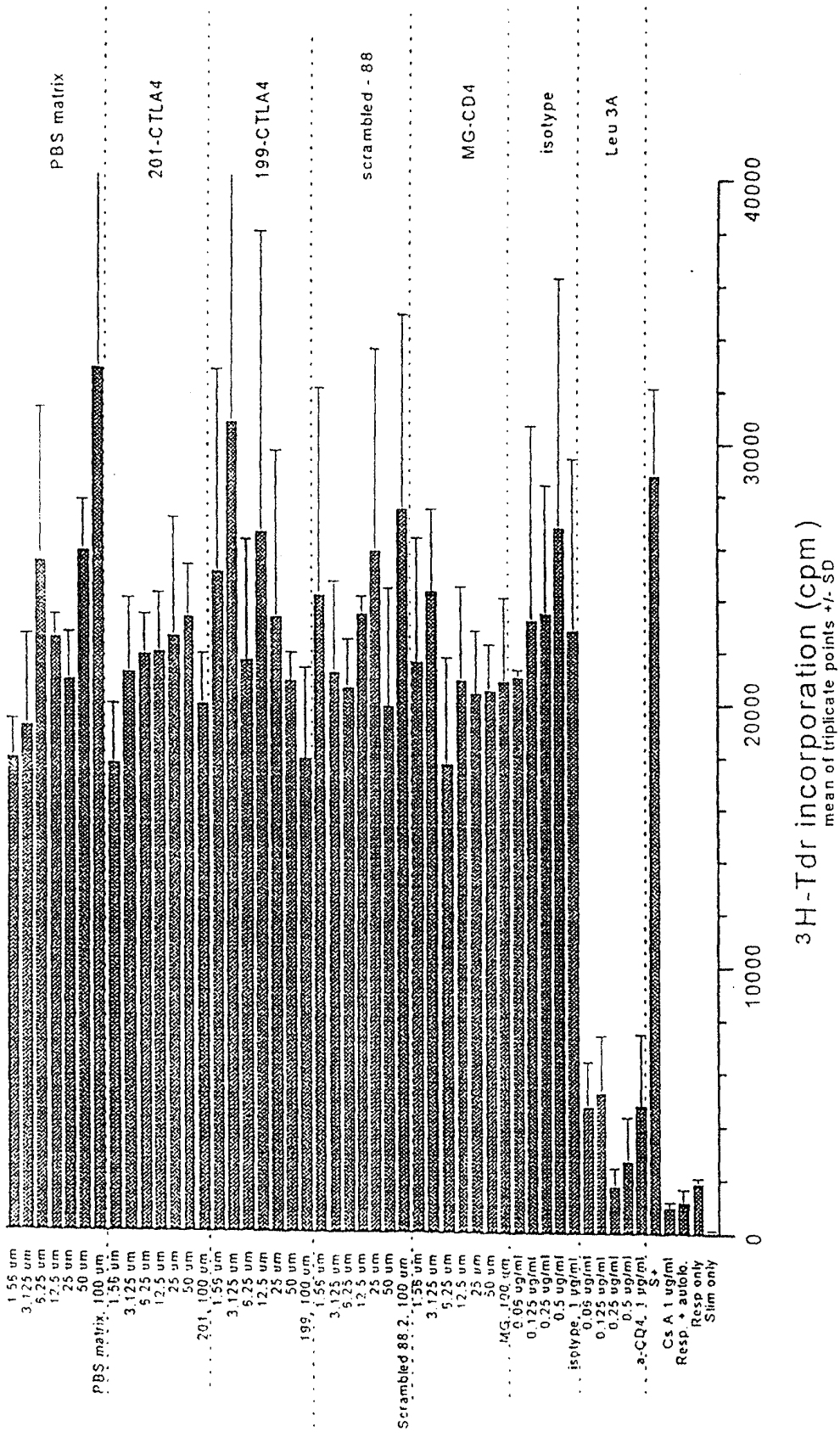
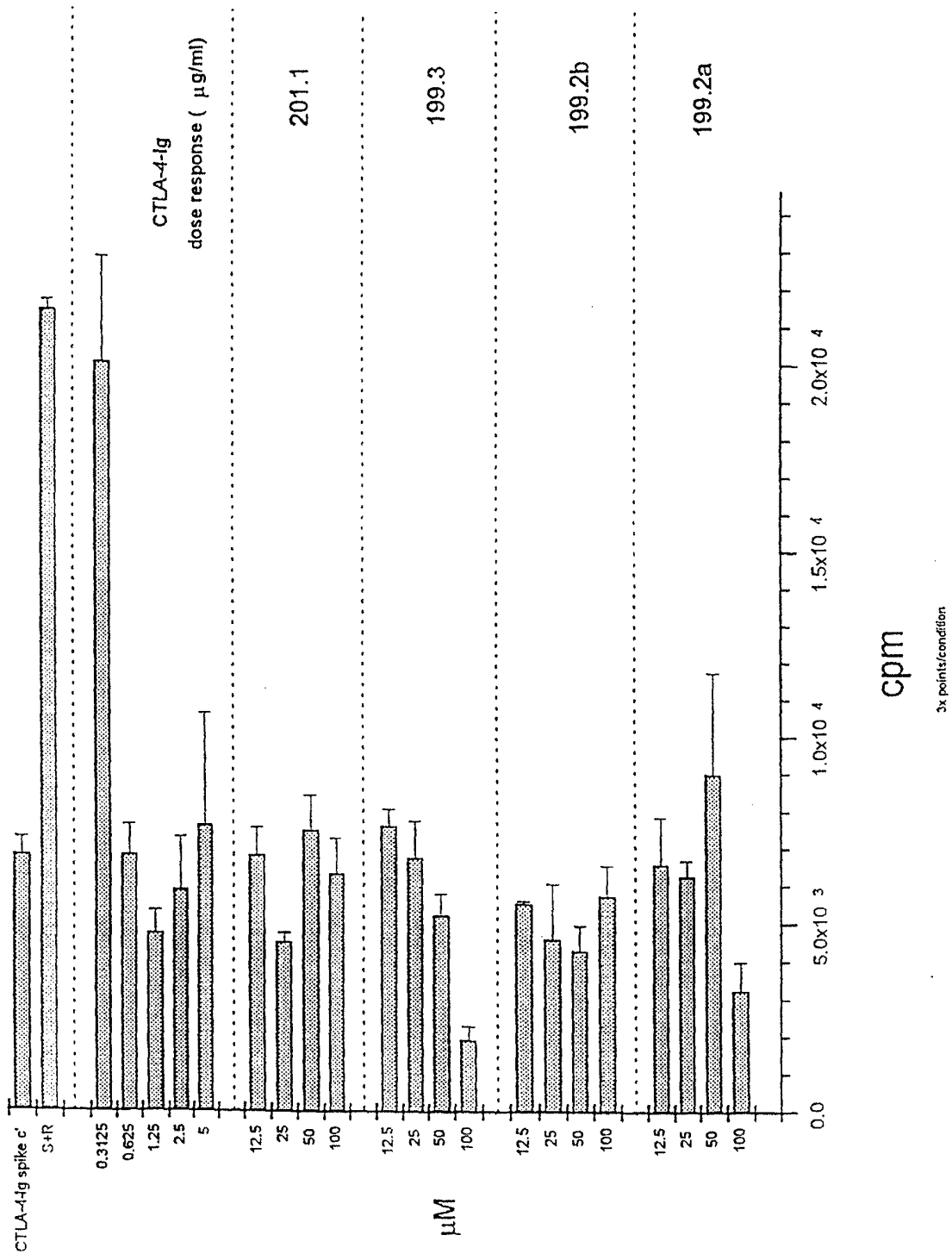

PBS matrix
201-CTLA4
199-CTLA4
scrambled - 88
MG-CD4
isotype
Leu 3A
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
PBS matrix 100 um
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
201 100 um
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
199 100 um
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
Scrambled 88.2 100 um
1.56 um
3.125 um
6.25 um
12.5 um
25 um
50 um
MG 100 um
0.05 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
isotype 1 ug/ml
0.05 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
a-CD4 1 ug/ml
S+
Cs A 1 ug/ml
Resp + autolo.
Resp only
Stim only
0
10000
20000
30000
40000
3H-Tdr incorporation (cpm)
mean of triplicate points +/- SD

FIG. 8


peptides assayed in the presence of 0.5 µg/ml CTLA-4-Ig
CTLA-4-Ig
dose response (µg/ml)
201.1
199.3
199.2b
199.2a
CTLA-4-Ig spike c'
S+R
0.3125
0.625
1.25
2.5
5
12.5
25
50
100
µM
0.0
5.0x10 3
1.0x10 4
1.5x10 4
2.0x10 4
cpm
3x points/condition

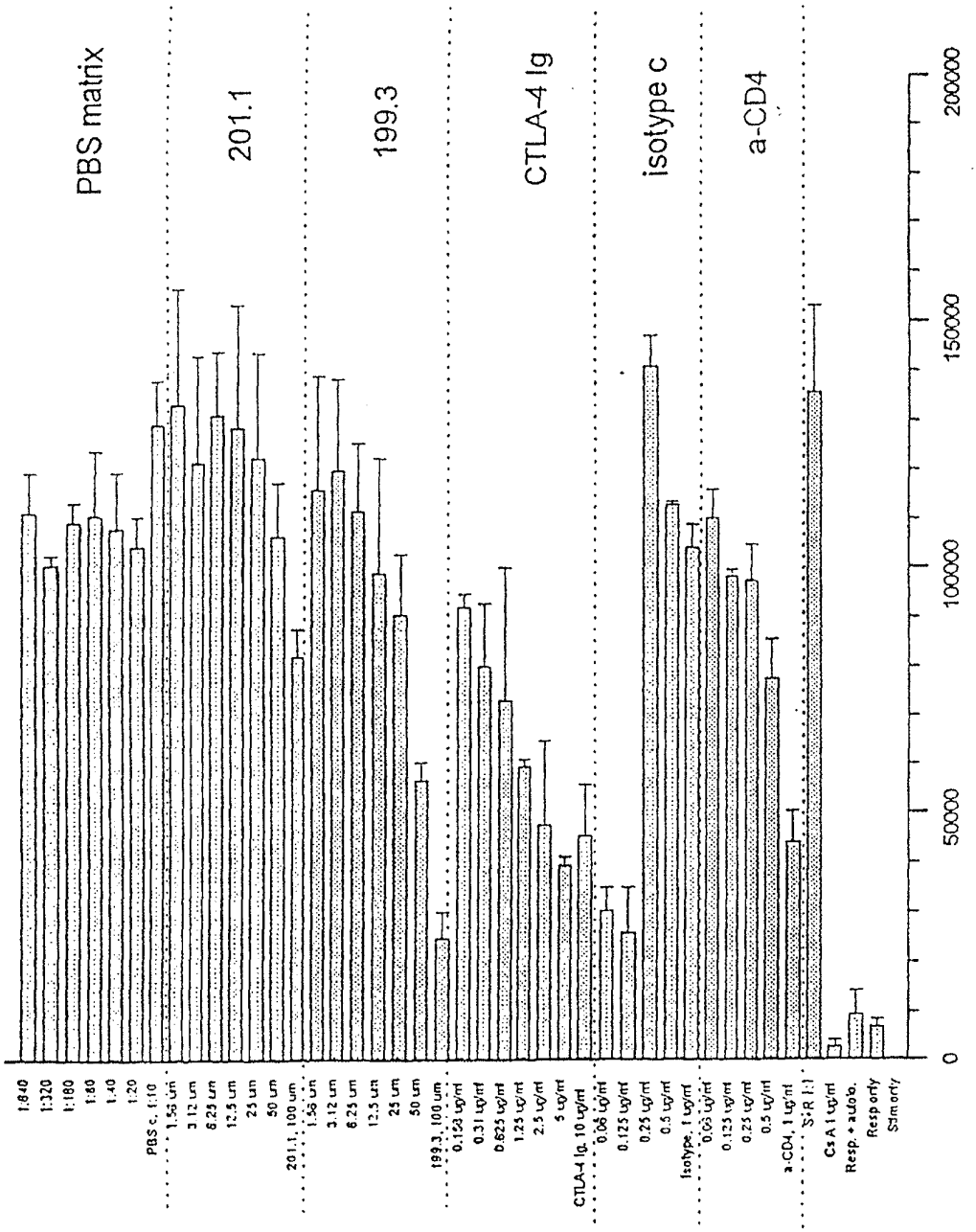
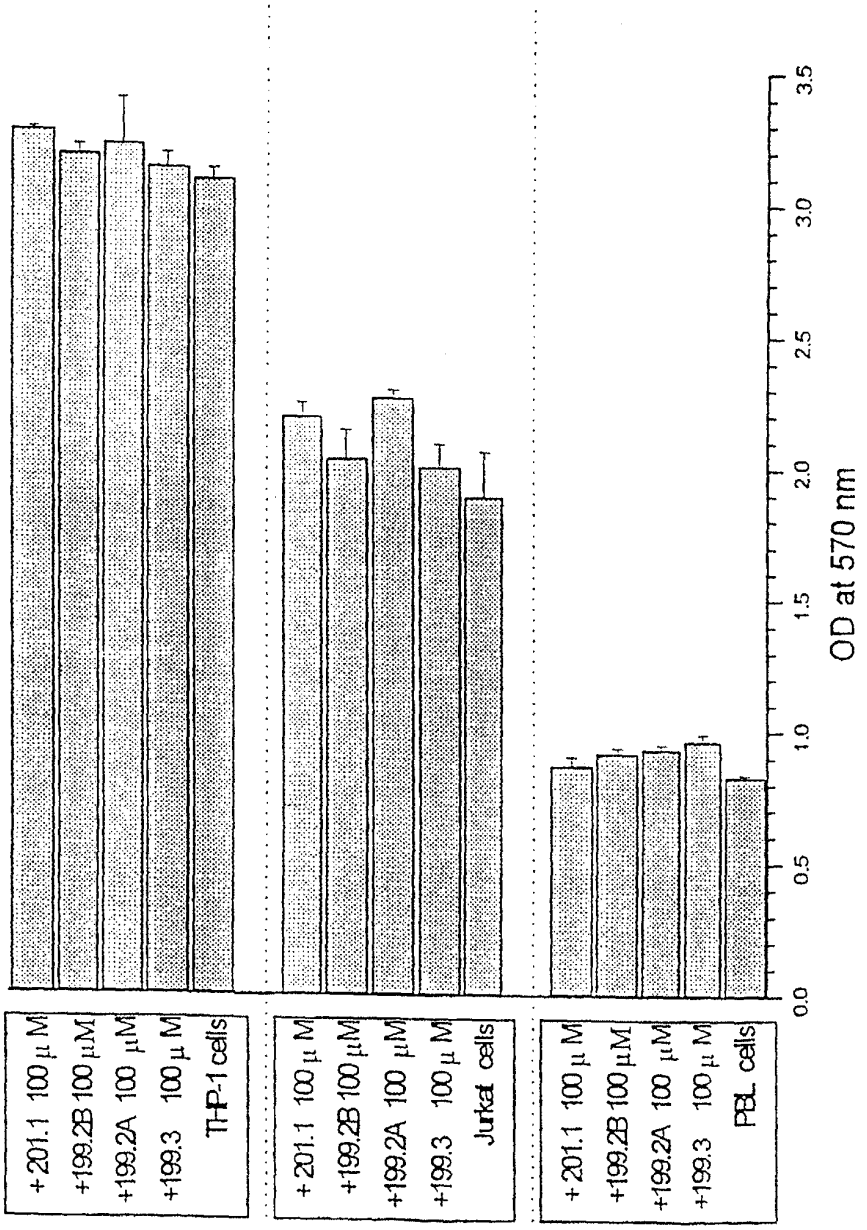

PBS matrix
201.1
199.3
CTLA-4 Ig
isotype c
a-CD4
0
50000
100000
150000
200000
^{3}H-Tdr incorporation (cpm)
mean of triplicate points +/- SD
1:640
1:320
1:160
1:80
1:40
1:20
PBS c. 1:10
1.56 um
3.12 um
6.25 um
12.5 um
25 um
50 um
201.1 100 um
1.56 um
3.12 um
6.25 um
12.5 um
25 um
50 um
199.3 100 um
0.156 ug/ml
0.31 ug/ml
0.625 ug/ml
1.25 ug/ml
2.5 ug/ml
5 ug/ml
CTLA-4 Ig 10 ug/ml
0.06 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
Isotype 1 ug/ml
0.06 ug/ml
0.125 ug/ml
0.25 ug/ml
0.5 ug/ml
a-CD4 1 ug/ml
SFR 1:1
CsA 1 ug/ml
Resp. + autol.
Resp only
Stim only

FIG. 9

Figure 10


OD at 570 nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
+ 201.1 100 µ M
+199.2B 100 µM
+199.2A 100 µM
+199.3 100 µ M
THP-1 cells
+ 201.1 100 µ M
+199.2B 100 µM
+199.2A 100 µM
+199.3 100 µ M
Jurkat cells
+ 201.1 100 µ M
+199.2B 100 µM
+199.2A 100 µM
+199.3 100 µ M
PBL cells

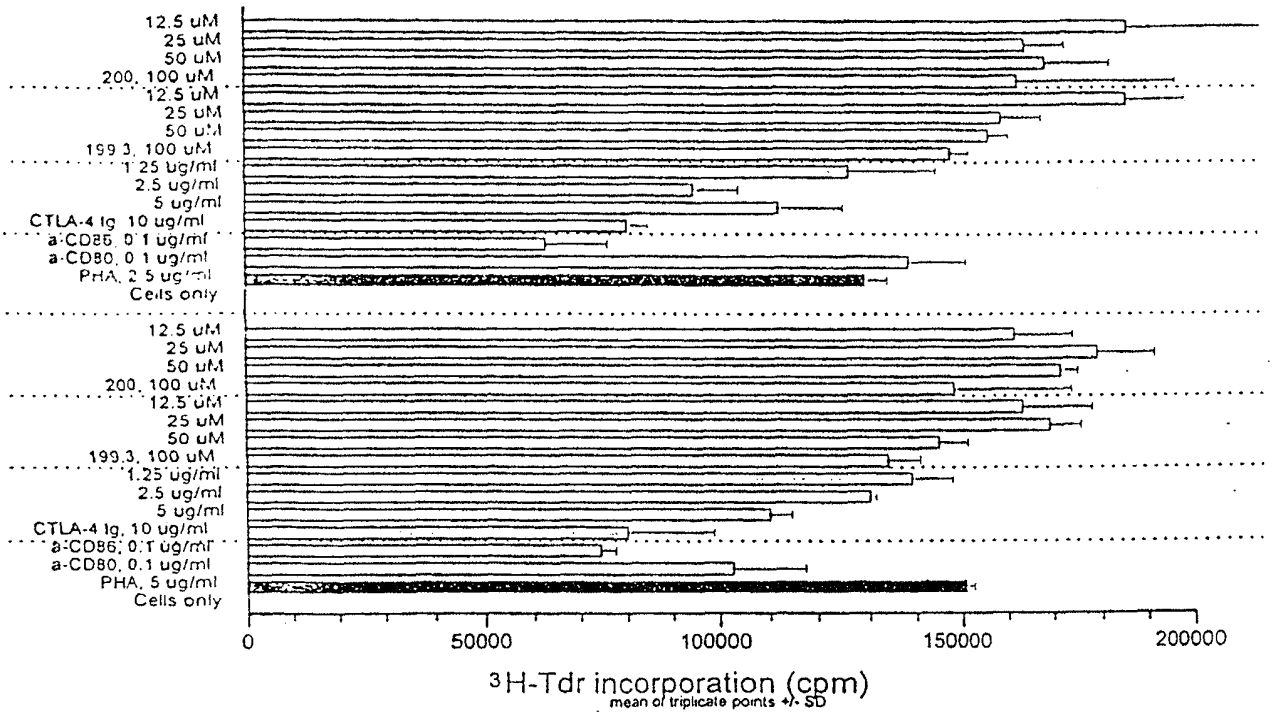

12.5 uM
25 uM
50 uM
200, 100 uM
12.5 uM
25 uM
50 uM
199.3, 100 uM
1.25 ug/ml
2.5 ug/ml
5 ug/ml
CTLA-4 Ig 10 ug/ml
a-CD86, 0.1 ug/ml
a-CD80, 0.1 ug/ml
PHA, 2.5 ug/ml
Cells only
12.5 uM
25 uM
50 uM
200, 100 uM
12.5 uM
25 uM
50 uM
199.3, 100 uM
1.25 ug/ml
2.5 ug/ml
5 ug/ml
CTLA-4 Ig, 10 ug/ml
a-CD86, 0.1 ug/ml
a-CD80, 0.1 ug/ml
PHA, 5 ug/ml
Cells only
0
50000
100000
150000
200000
3H-Tdr incorporation (cpm)
mean of triplicate points +/- SD

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12312

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/04, 38/12; C07K 7/04, 7/64,
US CL :514/9, 14, 15, 16, 17, 885; 530/317, 327, 328, 329

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/9, 14, 15, 16 , 17, 885; 530/317, 327, 328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WPIDS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,192,746 A (LOBL ET AL.) 09 March 1993, see entire document.	1-22
Y	EP 0757099 A2 (BRISTOL-MYERS SQUIBB COMPANY) 05 February 1997, see entire document.	1-22
Y	WO 93/00431 (BRISTOL-MYERS SQUIBB COMPANY) 07 January 1993, see entire document.	1-22
Y	HARPER et al. CTLA-4 and CD28 Activated Lymphocyte Molecules are Closely Related in Both Mouse and Human as to Sequence, Message Expression, Gene Structure, and Chromosomal Location. J. Immunol. August 1991, Vol. 147, pages 1037-1044, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1998	14 SEP 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	EVELYN RABIN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196